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# Sperm Mitochondrial DNA Biomarkers as a Measure of Male Fecundity and Overall Sperm Quality

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# Sperm Mitochondrial DNA Biomarkers as a Measure of Male Fecundity and Overall Sperm Quality

A Thesis Presented

by

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ABSTRACT

SPERM MITOCHONDRIAL DNA BIOMARKERS AS A MEASURE OF MALE FECUNDITY AND OVERALL SPERM QUALITY

MAY 2020

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**Introduction.** Sperm parameter analysis is the standard method of male fecundity testing; however, minimal evidence supports associations between individual sperm parameters and reproductive outcomes. Our previous work shows strong associations between sperm mitochondrial DNA copy number (mtDNAcn) and time-to-pregnancy (TTP) in general populations, and between mtDNAcn and fertilization outcomes in clinical populations. Thus it is possible for sperm mtDNA biomarkers to act as summary measures of semen quality. In this study, we developed a sperm quality index (SQI) from semen parameters and compared its ability to measure fecundity to sperm mtDNAcn.

**Methods.** We received 384 semen samples from the Longitudinal Investigation of Fertility in the Environment Study. Sperm mtDNAcn and mtDNA deletions (mtDNAdel) were quantified using a triplex probe-based qPCR method. The SQI was developed by ranking and summing select sperm parameters within the study population, including sperm concentration, sperm count, normal morphology, high DNA stainability, and DNA fragmentation to create a cumulative index. Discrete-time proportional hazards models

were used to determine fecundability odds ratios (FOR), indicating associations between mtDNAcn, SQI, and TTP. Receiver operating characteristic (ROC) analyses determined the validity of the SQI and mtDNAcn as predictors of pregnancy within 12 months.

**Results.** The SQI was highly associated with mtDNAcn, both continuously (Spearman Rho: -0.487; p-value: <0.001) and in deciles (ANOVA p-value: <0.001). The SQI (FOR: 1.25; 95% confidence interval (CI): 1.09, 1.43) and mtDNAcn (FOR: 0.754; 95% CI: 0.657, 0.866) performed similarly in discrete-time survival models and indicated a significant decrease and increase in TTP, respectively. MtDNAcn more effectively predicted pregnancy within 12 months (AUC: 0.703; 95% CI: 0.617, 0.789) than the SQI (AUC: 0.642; 95% CI: 0.531, 0.753). With multiple predictors, mtDNAcn outperformed summary models, with addition of the SQI and percent normal morphology minimally increasing model efficacy (AUC: 0.718, 95% CI: 0.617, 0.819).

**Conclusion.** The association between the SQI and mtDNAcn suggest that mtDNAcn may serve as a summary biomarker for overall sperm quality. Neither individual nor summed sperm parameters are useful indicators of couple fecundity and reproductive outcomes compared to mtDNAcn. These results suggest that mtDNAcn has potential for use as a biomarker of fecundity.

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# CHAPTER 1

# INTRODUCTION

Infertility effects approximately 15% of couples globally (Jungwirth & Giwercman et al., 2012) and is defined as the inability of a sexually active couple to achieve pregnancy within 12 months. Clinically, male infertility is determined using semen quality measures defined by World Health Organization (WHO) cut points (Cooper et al., 2010). Although they are used as the standard measurement of fertility, semen parameters have been shown to poorly predict reproductive outcomes and have a minimal association with fecundability (Buck Louis et al., 2014; Jungwirth & Giwercman et al., 2012). These results emphasize the importance of determining other potential biomarkers for male infertility that can better predict reproductive outcomes in both clinical and general populations.

Recent studies have determined associations between mitochondrial DNA (mtDNA) biomarkers and couple fecundity. Elevated mitochondrial DNA copy number (mtDNAcn) and mitochondrial DNA deletions (mtDNAdel) were recently associated with lower odds of fertilization in a cohort of couples undergoing fertility treatment (Wu et al., 2019). This study suggested that mtDNA biomarkers provided an incremental predictive ability of fertilization that was independent of semen parameters (Wu et al., 2019). Additionally, increased sperm mtDNAcn has been associated with a decrease in sperm parameters including sperm concentration, sperm count, and progressive motility (Song & Lewis, 2008; Zhang et al., 2016).

While there is no known mechanism for the association between mtDNA biomarkers, semen parameters, and couple fecundity, the characteristics of mitochondria and their importance for sperm motility provide motivation for their study in greater depth (Rajender et al., 2010). During spermatogenesis, mitochondria form tight helices at the midpiece of the sperm and sperm mtDNAcn is reduced by approximately eight to ten fold (Hecht et al., 1984; St John et al., 2010). It is possible that an error in spermatogenesis results in an increase in mtDNAcn and a decrease in fertilization ability due to the maternal nature of mitochondrial inheritance and the specialized defenses deployed to prevent paternal mtDNA inheritance (Al Rawi et al., 2011; St John et al., 2005; Sutovsky et al., 1999).

The search for alternative biomarkers for male fertility bears importance because of the recently determined global decline in semen parameters. A meta-regression analysis determined that total sperm count and sperm concentration have decreased over 50% since 1973 among men in developed countries (Levine et al., 2017). The purpose of this study was to use data collected from a general population prospective cohort to determine if a developed sperm quality index (SQI) could perform as well as mtDNAcn as a measure of couple fecundability and as a predictor for reproductive outcomes. As there is minimal correlation between individual semen parameters and time-to-pregnancy (TTP) (Buck Louis et al., 2014), the SQI consisted of multiple semen parameters combined into one cumulative parameter for sperm quality based on WHO dictated cut-points (Cooper et al., 2010).

# CHAPTER 2

# METHODS

## 2.1 Study Population

Whole semen samples were provided by the Longitudinal Investigation of Fertility in the Environment (LIFE) Study. Details for this study have been previously published (Buck Louis et al., 2014). In sum, 501 couples attempting to achieve pregnancy were randomly recruited in 16 select counties of Michigan and Texas in the United States between 2005 and 2009 (Buck Louis et al., 2014). Eligibility criteria included: 1) in a committed relationship and attempting to become pregnant; 2) no contraceptive use for >2 months and no injectable contraceptive use in the past year; 3) female partners age 18-40 and male partners 18 years or older; 4) female's menstrual cycles between 21-42 days; 5) an ability to communicate in English or Spanish; 6) no history of prior infertility treatment (Buck Louis et al., 2014). Study participants gave informed consent and human subjects approval was received from collaborating institutions before data collection. The current study consisted of 391 couples with an aliquot of semen available for mtDNA quantification and analysis. Seven samples were excluded due to limited sperm DNA concentration, resulting in a sample size of 384 couples.

## 2.2 Data Collection

Whole semen samples were collected in the homes of participating males, as previously published (Buck Louis et al., 2014). Briefly, males provided a baseline sample at study entry and a second sample one month later via masturbation (Buck Louis et al., 2014). Males were abstinent 2 days prior to sample collection and no lubricants were

used (Buck Louis et al., 2014). A detailed account of sperm parameter analysis is previously published, including details on the use of computer assisted semen analysis (CASA) and the Sperm Chromatin Structure Assay (SCSA) (Buck Louis et al., 2014). Time-to-pregnancy (TTP) was defined as the number of completed menstrual cycles before a human chorionic gonadotropin (hCG) confirmed pregnancy was determined by at-home pregnancy kits provided to participants (Buck Louis et al., 2014).

### 2.3 mtDNA Quantification

Sperm DNA was isolated from whole semen samples (n = 391) according to our previously published method (Wu et al., 2015). A one-step 50% gradient centrifugation was used to separate sperm from seminal plasma and somatic cells. Sperm was later homogenized with 0.2 mm steel beads in RLT buffer (Qiagen, Hilden, Germany) containing 50 mM tris(2-carboxyethyl)phosphine (TCEP; Pierce, Rockford, IL) and DNA was extracted via silica-column purification. MtDNA quantification was performed using a triplex probe-based qPCR assay for which details have been previously published (Huffman et al., 2018; Phillips et al., 2014). Mitochondrial DNA copy number (mtDNAcn) and mitochondrial DNA deletions (mtDNAdel) were simultaneously quantified using target regions in the D-Loop minor arc and in the major arc, respectively, using a nuclear RNase P target for reference (Huffman et al., 2018; Phillips et al., 2014).

### 2.4 Semen Quality Index Development

R software (R Foundation for Statistical Computing, Vienna, Austria) was used to assign a rank to select semen parameters with a value from 1 to 384 within the study

population. Samples with the same rank order were assigned the mean value, resulting in half measures. Semen parameters were chosen based on those outlined by World Health Organization (WHO) semen quality cutpoints (Cooper et al., 2010). Total sperm count, sperm concentration, percent normal morphology, percent DNA fragmentation, and percent high DNA stainability were ultimately chosen to compose the index. Sperm motility was excluded from the index because sperm were frozen for shipping before initial sperm motility analysis could be performed (Buck Louis et al., 2014), potentially having a negative impact on motility measurement. Following ranking within the study population, ranks for each individual parameter were then summed to compose a total sperm ranking or sperm quality index (SQI). Total sperm count, sperm concentration, and percent normal morphology were ranked from lowest to highest because a higher value in these parameters is considered higher quality semen (Cooper et al., 2010). Percent DNA fragmentation and percent high DNA stainability were ranked from highest to lowest, because high values of these parameters have been associated with undesirable fertility outcomes, including increased rates of early abortion and decreased sperm vitality among other parameters (Yang et al., 2019). Following assignment of a rank, the values per participant were summed to create an SQI that accounted for the combination of these parameters.

An SQI was also generated for the Sperm Environmental Epigenetics and Development (SEEDS) Study, a clinical cohort of couples seeking pregnancy through assisted reproductive technologies (ART) at Baystate Medical Center in Springfield, Massachusetts, USA (Wu et al., 2019). Inclusion criteria for SEEDS were 1) male partners between ages 18 – 55 years old with no history of vasectomy; 2) female partners

between 18 – 44 years old; 3) expected delivery at Baystate Medical Center; 4) fresh ejaculate sperm sample used for treatment (Wu et al., 2019). The same SQI was developed as was for the samples from the LIFE cohort, however percent motile sperm was included as a parameter in the SQI for SEEDS samples due to the availability of fresh non-frozen ejaculate for analysis, while excluded from the SQI for LIFE samples. Additionally, percent DNA fragmentation and percent high DNA stainability data were unavailable for SEEDS participants and were not included in the SQI.

**2.5 Statistical Analysis**

Participant characteristics were compared by quartile of mtDNAcn using ANOVA or chi-square as appropriate to determine relations. Spearman correlation analysis was used as a test for trend. MtDNAcn was right skewed and log (base 10) transformed to meet model assumptions. MtDNAdel distribution was approximately normal and thus was not transformed. Spearman correlation analysis was used to determine associations between mtDNA biomarkers and individual sperm parameters and the developed SQI. SQI was analyzed by decile of mtDNAcn using non-parametric ANOVA and Spearman correlation for continuous trend.

Discrete-time proportional hazards models were used to determine fecundability odds ratios (FOR) and determine associations between mtDNA biomarkers, SQI, and individual sperm parameters with TTP. Unadjusted parameters and z-score adjusted parameters were used. When adjusted for covariates such as age, BMI, race and ethnicity, and smoking, no variables were observed to represent confounders and thus models were run without covariates throughout the study. FOR estimates < 1.0 indicate a lower probability of pregnancy and thus longer TTP, while FOR estimates > 1.0 indicate higher

probability of pregnancy and shorter TTP. Models were adjusted for left truncation and 7 participants were excluded from the study for having a TTP of 0 yet never achieving pregnancy, indicating a loss to follow-up and resulting in a final sample size of 377 for statistical analysis.

The predictive ability of mtDNA biomarkers, the SQI, and individual sperm parameters for classifying couple fecundity was evaluated using receiver operating characteristic (ROC) curve analysis. Area under the curve (AUC) was used to quantify predictive ability in bivariate and multivariate analyses. Models were run using individual parameters and using combinations of parameters in order to distinguish those with the greatest predictive ability.

Spearman correlation analysis, ANOVA, and chi-square were performed using R (R Foundation for Statistical Computing, Vienna, Austria), while discrete-time proportional hazards models and ROC curve analysis were performed using SAS (SAS Institute, Cary, North Carolina, USA)

# CHAPTER 3

# RESULTS

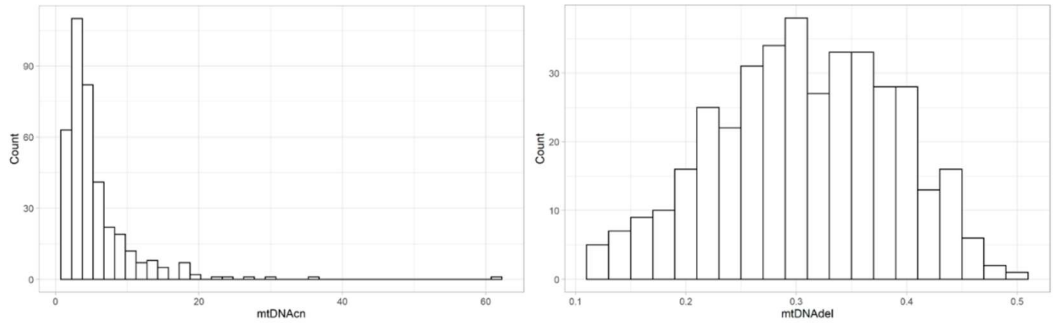


Figure 1. Distribution of sperm mtDNA biomarkers among LIFE male participants (n = 384).

Sperm mtDNAcn was right skewed and log (base 10) transformed to fit model assumptions in later analyses, while mtDNAdel fit an approximately normal distribution and was not transformed (Figure 1). Participant demographics by quartiles of sperm mtDNAcn can be seen in Table 1. Other than mtDNAdel ($p<0.001$), no characteristics were significantly associated with mtDNAcn.

Table 1. LIFE Study participants (n = 384) by quartile of mitochondrial DNA copy number (mtDNAcn).

| | Q1 | Q2 | Q3 | Q4 | | |
|---|---|---|---|---|---|---|
| Sperm mtDNAcn | (0.80 – 2.60) | (2.61 – 3.96) | (3.98 – 6.38) | (6.40 – 61.94) | | |
| | | Mean (SD) | | | p-value[1] | p-trend[2] |
| Male age | 31.8 (4.1) | 31.6 (5.0) | 32.1 (4.7) | 31.4 (5.2) | 0.73 | 0.53 |
| Male BMI[a] | 30.2 (5.6) | 29.5 (4.9) | 29.7 (6.0) | 30.3 (6.5) | 0.80 | 0.98 |
| Sperm mtDNAdel | 0.27 (0.09) | 0.29 (0.07) | 0.33 (0.06) | 0.37 (0.06) | <0.001 | <0.001 |
| Female age | 29.6 (3.6) | 30.3 (4.5) | 30.0 (3.9) | 29.8 (4.3) | 0.92 | 0.80 |
| Female BMI[b] | 28.2 (8.5) | 26.4 (6.5) | 26.9 (7.2) | 28.4 (7.9) | 0.77 | 0.56 |
| | | N (%) | | | | |
| Male current smoking[c] | 19 (19.8) | 16 (16.7) | 26 (27.1) | 21 (21.9) | 0.35 | 0.14 |

| | | | | | | |
|---|---|---|---|---|---|---|
| Male race[d] | | | | | 0.69 | 0.70 |
| White | 80 (83.3) | 76 (79.2) | 75 (78.1) | 80 (83.3) | | |
| Non-white | 15 (15.6) | 19 (19.8) | 21 (21.9) | 16 (16.6) | | |
| Female parity[e] | | | | | 0.87 | 0.71 |
| Never pregnant | 45 (46.9) | 37 (38.5) | 41 (42.7) | 44 (45.8) | | |
| Pregnant, no birth | 8 (8.3) | 7 (7.3) | 10 (10.4) | 9 (9.4) | | |
| Pregnant, live birth | 43 (44.8) | 51 (53.1) | 45 (46.9) | 43 (44.8) | | |

Abbreviations and Notes: BMI, body mass index; mtDNAdel, mitochondrial DNA deletions; [1] p-value determined by ANOVA for continuous variables and chi-square for categorical variables; [2] p-trend determined by Spearman correlation analysis; [a]Missing n = 3; [b]Missing n = 1; [c]Smoking status determined by a serum cotinine > 10 ng/mL; Missing n = 4; [d]Missing n = 2; [e]Missing n = 1

Two versions of the SQI were developed and both behaved similarly in models. The SQI consisting of total sperm count, sperm concentration, and percent normal morphology based on WHO standards had a minimum of 41.5 and maximum of 1,085. The SQI with percent DNA fragmentation and percent high DNA stainability added had a minimum of 96.5 and maximum of 1,786. Both SQI (Spearman Rho: -0.491; p-value: <0.001) and SQI including fragmentation and stainability (Spearman Rho: -0.487; p-value: <0.001) were highly associated with mtDNAcn. Both indices were also associated with mtDNAdel, (Spearman Rho: -0.271; p-value: <0.001 without fragmentation and stainability; Spearman Rho: -0.273; p-value: <0.001 with fragmentation and stainability) albeit to a smaller magnitude. Complete Spearman correlation analysis between individual parameters and mtDNA biomarkers is detailed in Table 2.

Table 2. Spearman Rank Correlation between Individual Sperm Parameters and Sperm MtDNA biomarkers (n = 384).

| | mtDNAcn | mtDNAdel |
|---|---|---|
| Semen Volume (mL) | 0.077 | 0.056 |
| Sperm Concentration ($10^6$/mL) | -0.502*** | -0.283*** |
| Total Sperm Count ($10^6$/mL*vol) | -0.419*** | -0.247*** |
| Percent of Live Sperm (%) | -0.156** | -0.111* |
| Average Path Velocity (μm/sec) | -0.136** | -0.073 |
| Straight Line Velocity (μm/sec) | -0.138** | -0.086 |
| Curvilinear Velocity (μm/sec) | -0.101* | -0.056 |
| Amplitude of Lateral Head (μm) | -0.088 | -0.079 |
| Beat Cross Frequency (Hz) | -0.041 | -0.022 |
| Straightness (%) | -0.090 | -0.050 |
| Linearity (%) | -0.121* | -0.057 |
| Percent Motility (%) | -0.327*** | -0.183*** |
| Sperm Head Length (μm) | 0.137** | 0.067 |
| Sperm Head Area ($\mu m^2$) | -0.032 | -0.069 |
| Sperm Head Width (μm) | -0.133** | -0.180*** |
| Elongation Factor (%) | -0.196*** | -0.153** |
| Sperm Head Perimeter (μm) | 0.083 | 0.037 |
| Sperm Head with Acrosome (%) | -0.168*** | -0.019 |
| Meeting of Strict Morphology Criteria (%) | -0.359*** | -0.173** |
| Meeting of WHO Morphology Criteria (%) | -0.366*** | -0.180*** |
| Amorphous (%) | 0.228*** | 0.154** |
| Round (%) | 0.254*** | 0.140** |
| Pyriform (%) | 0.074 | 0.031 |
| Bicephalic (%) | 0.189*** | 0.018 |
| Taper (%) | 0.081 | 0.027 |
| Megalohead (%) | 0.111* | 0.062 |
| Microhead (%) | 0.055 | 0.048 |
| Neck and Midpiece Abnormalities (%) | 0.303*** | 0.112* |
| Coiled Tail (%) | 0.220*** | 0.060 |
| Other Tail Abnormalities (%) | 0.283*** | 0.145** |
| Cytoplasmic Droplet (%) | 0.197*** | 0.097 |
| Immature Sperm (#) | 0.485*** | 0.206*** |
| DNA Fragmentation (%) | 0.215*** | 0.174*** |
| High DNA Stainability (%) | 0.264*** | 0.122* |
| | | |
| Sperm Quality Index | -0.491*** | -0.271*** |
| Sperm Quality Index including Fragmentation and Stainability[a] | -0.487*** | -0.273*** |

Abbreviations and Notes: mtDNAcn, mitochondrial DNA copy number; mtDNAdel, mitochondrial DNA deletions; [a]Missing n = 3;
*p < 0.05; **p < 0.01; ***p < 0.001

SQI including fragmentation and stainability was correlated with continuous mtDNAcn assessed via Spearman analysis and also with mtDNAcn in deciles (p-value: <0.001) (Figure 2). The SQI developed for the SEEDS cohort had a minimum of 9 and maximum of 681.5. Like for LIFE participants, SQI for SEEDS participants was highly

associated with continuous mtDNAcn (Spearman Rho: -0.517; p-value: <0.001) and mtDNAcn in deciles (p-value: <0.001) (Figure 2).

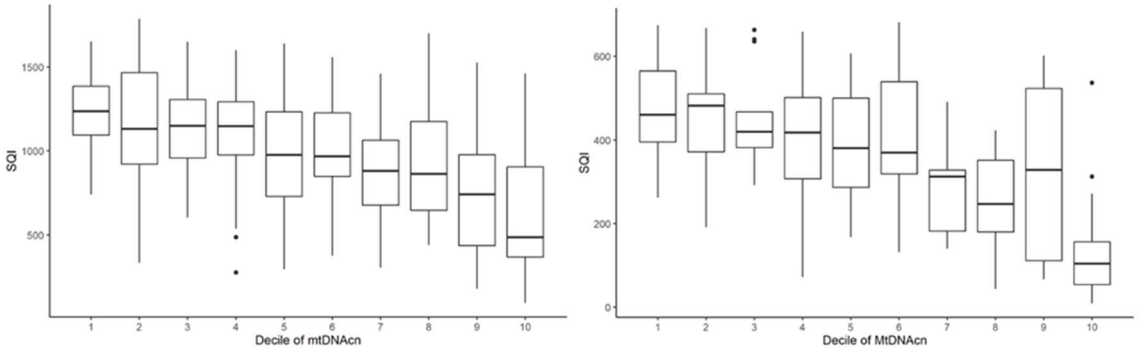


Figure 2. Relationship between generated SQI and mtDNAcn in LIFE (left; n=357) and SEEDS (right; n=140) cohorts. Continuous trend analyzed by Spearman Correlation (LIFE: Rho: -0.487, $p < 0.001$; SEEDS: Rho: -0.508, $p < 0.001$) and differences between deciles analyzed using non-parametric ANOVA (LIFE: $p < 0.001$; SEEDS: $p < 0.001$).

Discrete-time proportional hazards models revealed a strong inverse association between increasing mtDNAcn and TTP (FOR: 0.754; 95% CI: 0.657, 0.866). SQI including fragmentation and stainability was more strongly associated with TTP (FOR: 1.25 95% CI: 1.09, 1.43) than SQI without these two parameters (FOR: 1.20; 95% CI: 1.05, 1.38), however both associations were significant. Survival analyses for individual parameters used in SQI development can be found in Table 3, with models run using unadjusted values and z-score transformed values to account for differences of scale between measures. Percent normal morphology was also found to be highly associated with TTP, exhibiting an association of similar magnitude to the SQI with fragmentation and stainability (FOR: 1.27; 95% CI: 1.11, 1.45). MtDNAdel was not found to be associated with TTP.

Table 3. Fecundability Odds Ratios (FOR) estimated using Cox Proportional Hazards Models relating time to pregnancy (TTP) to sperm mtDNA biomarkers, Sperm Quality Index, and specific sperm parameters.

| | Z-Scores | Untransformed Data |
|---|---|---|
| MtDNAcn | 0.754 (0.657, 0.866) *** | 0.380 (0.237, 0.611)*** |
| MtDNAdel | 0.908 (0.798, 1.034) | 0.306 (0.062, 1.509) |
| Sperm Quality Index | 1.204 (1.054, 1.376) ** | 1.001 (1.000, 1.001)** |
| Sperm Quality Index including DNA Fragmentation and Stainability | 1.249 (1.091, 1.430)** | 1.001 (1.000, 1.001)** |
| Sperm Concentration | 1.097 (0.976, 1.233) | 1.002 (1.000, 1.004) |
| Total Sperm Count | 1.171 (1.038, 1.321)* | 1.001 (1.000, 1.002)* |
| Meeting of WHO Morphology Criteria | 1.267 (1.111, 1.445)*** | 1.020 (1.009, 1.031)*** |
| Neck and Midpiece Abnormalities | 0.901 (0.789, 1.030) | 0.989 (0.976, 1.003) |
| DNA Fragmentation | 0.837 (0.727, 0.963)* | 0.983 (0.970, 0.996)* |
| High DNA Stainability | 0.872 (0.758, 1.005) | 0.974 (0.948, 1.001) |

Abbreviations and Notes: MtDNAcn, mitochondrial DNA copy number; MtDNAdel, mitochondrial DNA deletions; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

ROC analysis determined that mtDNAcn had higher predictive ability of pregnancy within 12 months (AUC: 0.703; 95% CI: 0.617, 0.789) than any other individual parameter and the SQI index both with (AUC: 0.642; 95% CI: 0.531, 0.723) and without (AUC: 0.650; 95% CI: 0.547, 0.753) DNA fragmentation and stainability. Surprisingly, total sperm count (AUC: 0.674; 95% CI: 0.576, 0.771) was the parameter with the strongest predictive ability other than mtDNAcn. However, total sperm count was not as strongly associated with TTP (FOR: 1.17; 95% CI: 1.04, 1.32) as morphology and the SQI, and was not explored further in later analyses.

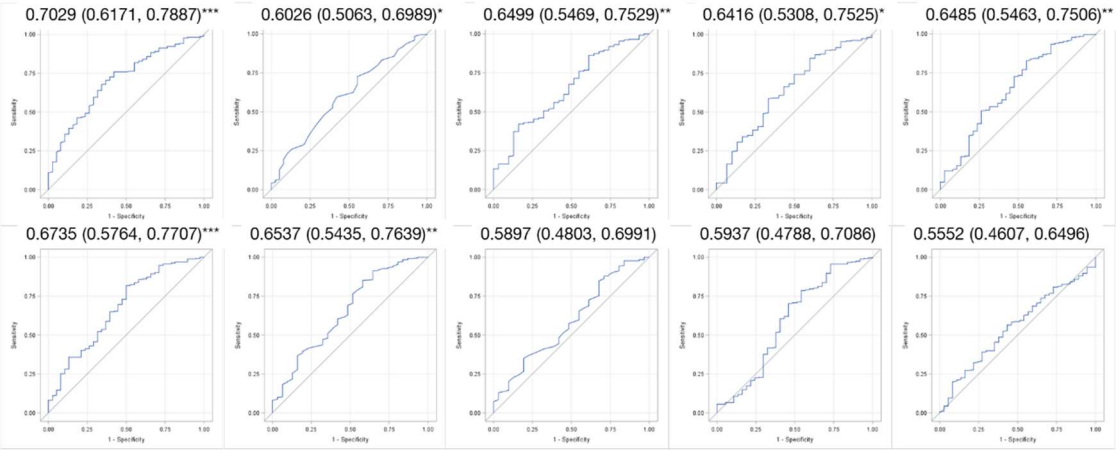


Figure 3. Receiver Operating Characteristic (ROC) analysis unadjusted for confounding variables using mtDNA, sperm quality index, and specific sperm parameters as a predictor for pregnancy within 12 months of trying.
Top row left to right: MtDNAcn; MtDNAdel; Sperm Quality Index; Sperm Quality Index including DNA Fragmentation and Stainability; Sperm Concentration
Bottom row left to right: Total Sperm Count; Meeting of WHO Morphology Criteria; Neck and Midpiece Abnormalities; DNA Fragmentation; High DNA Stainability
*p < 0.05; **p < 0.01; ***p < 0.001

Using previous results, ROC analyses were performed using multiple predictors in an attempt to determine the parameter best able to predict pregnancy within 12 months. MtDNAcn, percent normal morphology, and the SQI combined were shown to have the strongest predicting power (AUC: 0.718; 95% CI: 0.617, 0.819). However, when mtDNAcn was removed and the model consisted only of percent normal morphology and the SQI, a large decrease in predictive ability was observed (AUC: 0.661; 0.549, 0.774). Additionally, the model consisting of all three parameters exhibited a minimal increase in predictive ability in comparison to mtDNAcn alone (AUC: 0.703; 95% CI: 0.617, 0.789).

Table 4: Components of Models and Predictive Power of Each Model Suggested by AUC of ROC Curve.

| Model | mtDNAcn | Morphology | SQI | AUC (95% CI) |
|---|---|---|---|---|
| 1 | X | | | 0.703 (0.617, 0.789) |
| 2 | | X | | 0.654 (0.544, 0.764) |
| 3 | | | X | 0.642 (0.531, 0.753) |
| 4 | X | X | | 0.713 (0.615, 0.812) |
| 5 | X | | X | 0.700 (0.599, 0.801) |
| 6 | | X | X | 0.661 (0.549, 0.774) |
| 7 | X | X | X | 0.718 (0.617, 0.819) |

Abbreviations and Notes: SQI: Sperm Quality Index with DNA fragmentation and stainability; AUC: Area Under Curve; ROC: Receiver Operating Characteristic; X indicates parameter included in model.

# CHAPTER 4

# DISCUSSION

The sperm quality index (SQI) both with and without DNA fragmentation and high DNA stainability was highly associated with sperm mtDNAcn (p-value: <0.001) when assessed both continuously and in deciles (Table 2; Figure 2). While the parameters were highly correlated (Table 2; Figure S1; Figure S2), sperm mtDNAcn demonstrated a stronger predictive ability for pregnancy within 12 months than the SQI (Figure 3; Table 4). In magnitude, both mtDNAcn and the SQI were similarly and significantly associated with TTP, with an FOR of 0.754 (95% CI: 0.66, 0.87) for mtDNAcn and an FOR of 1.25 (95% CI: 1.09, 1.43) for the SQI (Table 3). Couples with an elevated sperm mtDNAcn exhibited longer TTP while those with an increased SQI exhibited shorter TTP.

While the summary SQI consisting of multiple semen parameters exhibited an association with couple fecundity (Table 3), individual semen parameters have shown poor associations with fecundability in previous work. In a comparison of semen analysis results between fertile and infertile couples, none of the individual parameters were observed to predict fertility status (Guzick et al., 2001). In this study, percent normal morphology exhibited the greatest discriminating ability, but still exhibited strong overlap between groups. These findings are consistent with our data in which percent normal morphology is associated with TTP (FOR: 1.267; 95% CI: 1.111, 1.445), but is not as useful a predictor of pregnancy within 12 months (AUC: 0.654; 95% CI: 0.544, 0.764) as sperm mtDNAcn (AUC: 0.703; 95% CI: 0.617, 0.789)(Table 3; Figure 3). Additionally, in a general population cohort study observing 36 different semen parameters and their association with TTP, only one parameter exhibited a significant

correlation in multivariable models, while individual model estimates were of extremely low magnitude (Buck Louis et al., 2014).

In addition to the correlation between mtDNAcn and TTP, previous work has established a strong association between mtDNAcn and fertilization rates in couples undergoing ART. In a cohort of 119 couples, higher sperm mtDNAcn and mtDNAdel were strongly associated with lower fertilization rates and embryo quality (Wu et al., 2019). Meanwhile, a meta-analysis reported that leukocytospermic men exhibited significant decreases in semen parameters, including progressive motility and sperm concentration, yet no decrease in fertilization or pregnancy rates were observed when these couples sought ART (Castellini et al., 2020). These results indicate a poor association between individual sperm parameters and fertilization outcomes in comparison to mtDNA biomarkers.

While the mechanism underlying the strong association between sperm mtDNAcn, semen parameters, and reproductive outcomes is unclear, it is possible that sperm mtDNAcn can act as a marker for abnormal spermatogenesis. During spermiogenesis, mtDNA is depleted eight to ten fold, thus it is possible that an increased sperm mtDNAcn results from an error in this phase of sperm maturation (Hecht et al., 1984; St John et al., 2010). The strong association observed between semen quality and sperm mtDNAcn observed in this study is consistent with this theory (Table 2; Figure S1; Figure S2). Furthermore, it is possible that the robust cellular defenses deployed to ensure maternal mtDNA inheritance (Al Rawi, et al., 2011, St John, et al., 2005, Sutovsky, et al., 1999) are partially responsible for the association between elevated sperm mtDNAcn and poor fertilization outcomes (Darr et al., 2017; Wu et al., 2019). There is also potential for

an elevated sperm mtDNAcn to act as a biomarker for oxidative stress due to the susceptibility of sperm mtDNA to oxidative damage because of the role of the mitochondrion in ATP production and its proximity to the electron transport chain (Phillips et al., 2014). mtDNA also lacks the robust repair mechanisms found in nuclear DNA (Phillips et al., 2014). The definitive mechanism remains elusive and further study is needed to fully support these notions and to determine the mechanistic link between sperm mtDNAcn, semen parameters, and couple fecundity.

# CHAPTER 5

# CONCLUSION

Individual semen parameters are ineffective measures of male fecundity and of couples' reproductive ability, thus determining alternative markers of overall reproductive health are of critical importance. Our study provides support to the notion that mtDNAcn can serve as an overall biomarker for semen quality and couple fecundity as it has a strong association with TTP and outperforms both individual semen parameters and our developed cumulative index in predictive ability for pregnancy within 12 months. This study asserts that sperm mtDNAcn could serve as a useful and more accurate single measure parameter for male reproductive health and pregnancy success, as well as overall semen quality, in the general population.

## APPENDIX A

## SUPPLEMENTAL FIGURES

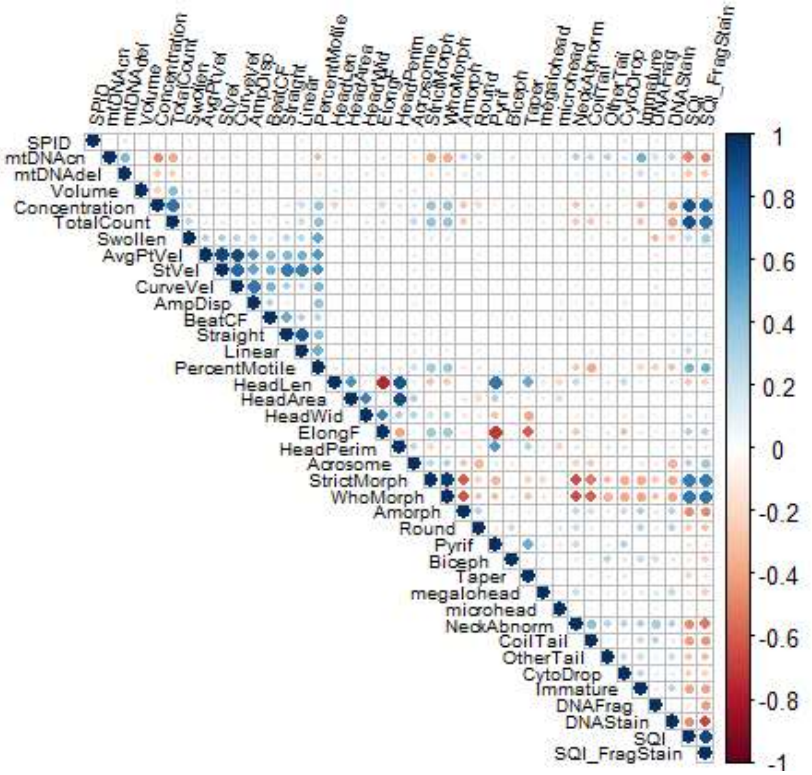


Figure 4. Heatmap indicating Spearman Rank correlation between mtDNA biomarkers, individual sperm parameters, and the sperm quality index with (SQI_FragStain) and without (SQI) DNA fragmentation and stainability. Colors indicate strength of correlation and size indicates significance. Significance level $p < 0.05$.

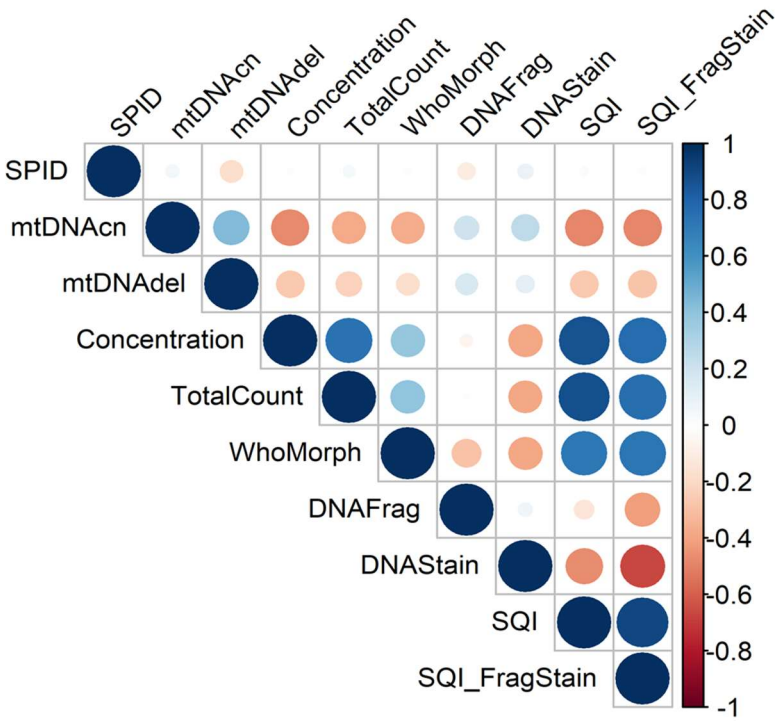


Figure 5. Heatmap indicating the Spearman Rank correlation between mtDNA biomarkers, sperm quality index with (SQI_FragStain) and without (SQI) DNA fragmentation and stainability, and individual sperm parameters used to build the SQI. Color indicates strength of correlation and size indicates significance. Significance level $p < 0.05$.

# APPENDIX B

# MITOCHONDRIAL SHEATH LENGTH MEASUREMENT PROTOCOL

## B.1 Introduction

Sperm mitochondria have a critical role in sperm motility and function, and thus have earned attention as potential indicators of male fertility (Rajender et al., 2010). During spermatogenesis, the rounded mitochondria elongate and form tight helices within the midpiece of the sperm, thus forming the mitochondrial sheath. Previous work has determined that a shorter mitochondrial sheath length correlates with lower fertilization outcomes in bull sperm (Sutovsky et al., unpublished work). Another study has determined the importance of glycerol kinase 2 in the formation of the sperm mitochondrial sheath (Shimada et al., 2019). When glycerol kinase 2 function was interrupted in mice, sperm mitochondrial sheath length formation during spermatogenesis was interrupted and resulted in an infertility phenotype in these mice, suggesting a link between proper sheath formation and fertilization ability (Shimada et al., 2019).

Our previous work has established a negative association between mtDNAcn and reproductive outcomes in both clinical populations (Wu et al., 2019) and in the general population (Rosati et al., unpublished work). These findings raise the question of what other sperm mitochondrial DNA biomarkers are associated with reproductive outcomes. As a result, our laboratory has sought to optimize a protocol for the measurement of sperm mitochondrial sheath length in human sperm.

## B.2 Protocol

### B.2.1 Processing

*Reagents:*

Quinn's Sperm Washing Medium (Cooper Surgical Inc., Trumbull, Connecticut, USA; Catalog no. ART-1006)

PureCeption 100% Isotonic Soluton (Cooper Surgical Inc., Trumbull, Connecticut, USA; Catalog no. ART-2100)

50% PureCeption Mix: 5 mL PureCeption mixed with 5 mL Quinn's Sperm Washing Medium

1. Allow whole semen to liquefy for 30 minutes at room temperature
2. Centrifuge semen at 500 g for 10 min
3. Remove and discard the seminal plasma supernatant
4. Resuspend the pellet in 1.5 mL of Quinn's Sperm Washing Medium
5. Layer the resuspended pellet carefully on top of 1 mL of 50% PureCeption mix
6. Centrifuge at 500 g for 25 min
7. Remove pellet and buffer to a final volume of 500 uL
8. Add pellet and buffer to 2 mL Quinn's Sperm Washing Medium
9. Mix by pipetting
10. Centrifuge at 500g for 5 min
11. Remove pellet and buffer to a final volume of 1 mL
12. Store in fridge for later use, if necessary

### B.2.2 Fixation

*Reagents:*

37% Formaldehyde (Thermo Scientific, Waltham, Massachusetts, USA)

10X PBS (Applied Biosystems, Foster City, California, USA)

1X PBS: 1 mL 10X PBS mixed with 9 mL deionized (DI) water

4% Formaldehyde: 500 uL 37% formaldehyde mixed with 4.5 mL 1X PBS

1. Add processed sperm dropwise in 1 mL 4% formaldehyde
2. Invert tube five times to mix
3. Incubate 30 min at room temperature
4. Centrifuge formaldehyde/sperm mixture at 600 g for 15 min
5. Pour off supernatant as much as possible and resuspend pellet in remainder
6. Add 1 mL 1X PBS to resuspend sample
7. Store in fridge for later use, if needed

### B.2.3 Counting

1. Dilute 3 uL of fixed sample in 27 uL of 1X PBS (dilution factor (DF) = 10)
2. Add 10 uL of diluted fixed sample to each side of the hemocytometer and let stand several minutes
3. Count five 0.2 mm by 0.2 mm squares on each side of hemocytometer
4. Sum counts and determine an average sperm count per side of the hemocytometer

5. Concentration = average * 5 (to represent squares not counted) * DF * 10,000 (volume of hemocytometer)

### B.2.4 Staining

*Reagents:*

10X PBS (Applied Biosystems, Foster City, California, USA)

1X PBS: 1 mL 10X PBS mixed with 9 mL DI water

Ethylenediaminetetraacetic acid (EDTA) (Fisher Scientific, Waltham, Massachusetts, USA)

Triton X-100 (Fisher Bioreagents, Waltham, Massachusetts, USA)

Hoechst 33342 Nuclear Stain (Enzo Life Sciences, Inc., Farmingdale, New York, USA; Catalog no. ENZ-51035-K100)

MitoView Green (Biotium, Fremont, California, USA; Catalog no. 70054)

Dimethyl Sulfoxide (DMSO) (Sigma Aldrich, St. Louis, Missouri, USA)

Permeabolizing Solution: 4.945 mL 1X PBS mixed with 30 uL EDTA and 25 uL Triton X-100

1. Centrifuge fixed sperm at 600 g for 15 min
2. Remove supernatant and resuspend pellet in remainder
3. Add resuspended pellet dropwise to 1 mL permeabolizing solution
4. Incubate on ice for 30 min

5. Centrifige at 600 g for 15 min
6. Remove supernatant and resuspend pellet in remainder
7. Add 500 uL 1X PBS to resuspended pellet and mix via pipetting
8. Centrifuge at 600 g for 15 min
9. During spin, prepare desired dye concentrations
    a. Hoechst 33342:
        i. add 1 uL to 99 uL 1X PBS
    b. MitoView Green:
        i. Make 200 uM stock by adding 50 ug dye to 400 uL DMSO
        ii. Create a 100 uM solution by diluting 200 uL of the 200 uM solution in 200 uL DMSO.
        iii. Add 1 uL of the 100 uM stock to 9 uL of DMSO to create a 10 uM solution.
10. Remove supernatant and resuspend pellet in 500 uL 1X PBS
11. Add 1 uL of each diluted stain to sperm
12. Incubate in dark box for 30 min at room temperature
13. Centrifuge at 600 g for 15 min
14. Remove supernatant and resuspend pellet in 50 uL of 1X PBS

**B.2.5 Polylysine Treatment:**

*Reagents:*

Poly-L-lysine (Sigma Aldrich, St. Louis, Missouri USA; Catalog no. P4707-50ML)

1. Using 1 mL poly-L-lysine per 25 $cm^2$, coat coverslips by pipetting onto coverslip and rocking the liquid back and forth to coat the entire surface
    a. Use 500 uL of poly-L-lysine
2. Overturn coverslips into excess poly-L-lysine for complete coating
3. Allow to soak for 5 min
4. Remove poly-L-lysine via aspiration
5. Rinse coverslips thoroughly with deionized (DI) water
6. Allow coverslips to dry for at least 2 hours

**B.2.6 Imaging:**

1. Add 10 uL stained sample to each slide and cover with poly-L-lysine treated coverslips
2. Tape coverslips to slide
3. Transfer to microscopy core in dark box to minimize light exposure
4. Place slide coverslip-down on microscope stage
5. Using 60x objective of A1-Resonant Scanning Confocal Microscope with TIRF Module (Nikon Instruments, Melville, New York, USA), locate and focus sperm
6. Use automated microscope function to image sperm in a 0.1 mm radius on slide area

### B.2.7 Data Analysis:

1. Use NIS-Elements AR software (Nikon Instruments, Melville, New York, USA) to detect midpieces of sperm cells based on fluorescence intensity of MitoView Green and proximity to Hoechst 33342 stain
2. Once detected, use template to measure midpieces at the longest point
3. Export sheath measurements to spreadsheet for future analysis

## B.3 Significance and Future Directions

When this protocol is fully optimized, it will be possible to use LIFE semen samples to determine any possible correlation between sperm mitochondrial sheath length and time-to-pregnancy (TTP) using discrete-time proportional hazards model. Additionally, it will be possible to test the functionality of sheath length as a predictor for couple fecundity by using receiver operating characteristic (ROC) analysis of mitochondrial sheath length with occurrence of pregnancy within 12 months. The potential for mitochondrial sheath length to serve as another biomarker for male and couple fecundity is of importance because of the poor association between sperm parameters and reproductive outcomes (Buck Louis et al., 2014; Wu et al., 2019; Guzick et al., 2001). If any association is determined, it would be possible to combine the predictive abilities of sperm mtDNAcn and mitochondrial sheath length to measure overall semen quality in the general population. However, for this study to continue, changes to the above protocol must be made.

In terms of imaging, problems with automated sheath detection are hindering results. The automated sheath detection template must be calibrated further to detect only

the midpiece of the sperm and to ignore any non-specific staining in other regions of the sperm or throughout the buffer. For this, it could be advisable to use a stained control, containing only buffer with no sperm, and determine an adequate method for eliminating background fluorescence from sample images. Rather than eliminating non-specific staining post-imaging, it could be possible to ensure more specific staining through the use of antibodies for mitochondrial detection in the staining process.

Cellular aggregates and non-specific staining are key issues with the automated detection template. The treatment of coverslips with poly-L-lysine has decreased aggregates within the sample by creating a single layer of cells, however aggregates are still present. It could be beneficial to use a well and three dimensional droplet of sample, rather than a flat coverslip, and to allow the sperm to settle into a single layer. This would require re-optimization of the imaging protocol because it is unknown how long the sample would need to rest in order for cells to settle into a single layer without the use of a coverslip. To eliminate aggregates without re-optimizing the protocol, post-processing filters can be deployed; however, circularity filters have been ineffective in removing undesirable aggregates and another method has yet to be determined.

Finally, to improve statistical power of these assessments, it is necessary to image more sperm and increase sample size. The automated imaging of the A1R-TIRF microscope (Nikon Instruments, Melville, NY) can only produce a limited number of images per slide, after which the stains are photo-bleached and a new slide must be prepared. Samples should likely be run in triplicate in order to obtain maximum sheath measurements due to limited imaging capacity and the necessary filters that eliminate select sperm cells from measurement.

While many changes remain to be made to this protocol, determining any existing correlation between mitochondrial sheath length and reproductive outcomes is of importance due to the unknown nature of idiopathic male infertility and the unknown mechanism underlying the association between mtDNAcn and male fecundity. If an association exists between mitochondrial sheath length and male fecundity, it could provide an underlying mechanism for male infertility such as abnormal spermatogenesis and disruption of sperm mitochondrial sheath formation (Shimada et al., 2019).

## BIBLIOGRAPHY


Al Rawi, S., Louvet-Vallee, S., Djeddi, A., Sachse, M., Culetto, E., Hajjar, C., Boyd, L., Legouis, R., & Galy, V. (2011). Postfertilization autophagy of sperm organelles prevents paternal mitochondrial DNA transmission. Science, 334(6059), 1144-1147. doi: 10.1126/science.1211878

Buck Louis, G.M., Schisterman, E.F., Sweeney, A.M., Wilcosky, T.C., Gore-Langton, R.E., Lynch, C.D., Boyd Barr, D., Schrader, S.M., Kim, S., Chen, Z., & Sundaram, R. (2011). Designing prospective cohort studies for assessing reproductive and developmental toxicity during sensitive windows of human reproduction and development--the LIFE Study. Paediatric and Perinatal Epidemiology, 25(5), 413-424. doi: 10.1111/j.1365-3016.2011.01205.x

Buck Louis, G., Sundaram, R., Schisterman, E., Sweeney, A., Lynch, C., Kim, S., Maisog, J., Gore-Langton, R., Eisenberg, M., & Chen, Z. (2014). Semen quality and time to pregnancy: the Longitudinal Investigation of Fertility and the Environment Study. Fertility and Sterility, 101(2), 453-462. doi:10.1016/j.fertnstert.2013.10.022

Castellini, C., D'Andrea, S., Martorella, A., Minaldi, E., Necozione, S., Francavilla, F., Francavilla, S., & Barbonetti, A. (2020). Relationship between leukocytospermia, reproductive potential after assisted reproductive technology, and sperm parameters: a systematic review and meta-analysis of case-control studies. Andrology, 8(1), 125-135. doi: 10.1111/andr.12662

Cooper, T., Noonan, E., Von Eckardstein, S., Auger, J., Gordon Baker, H., Behre, H., Haugen, T., Kruger, T., Wang, C., Mbizvo, M., & Vogelsong, K. (2010). World Health Organization reference values for human semen characteristics. Advanced Access publication, 16(3), 231-245. doi:10.1093/humupd/dmp048

Darr, C.R., Moraes, L.E., Connon, R.E., Love, C.C., Teague, S., Varner, D.D., & Meyers, S.A. (2017). The relationship between mitochondrial DNA copy number and stallion sperm function. Theriogenology, 94, 94-99. doi: 10.1016/j.theriogenology.2017.02.015

Guzick, D.S., Overstreet, J.W., Factor-Litvak,P., Brazil, C.K., Nakajima, S.T., Coutifaris, C., Carson, S.A., Cisneros, P., Steinkampf, M.P., Hill, J.A. Xu, D., & Vogel, D.L. (2001). Sperm morphology, motility, and concentration in fertile and infertile men. The New England Journal of Medicine, 345(19), 1388-1393. doi: 10.1056/NEJMoa003005

Hecht, N.B., Liem, H., Kleene, K.C., Distel, R.J., & Ho, S.M. (1984). Maternal inheritance of the mouse mitochondrial genome is not mediated by a loss or gross alteration of the paternal mitochondrial DNA or by methylation of the oocyte mitochondrial DNA. Developmental Biology, 102(2), 452-461. doi: 10.1016/0012-1606(84)90210-0

Huffman, A., Wu, H., Rosati, A., Rahil, T., Sites, C., Whitcomb, B., & Pilsner, R.J. (2018). Associations of urinary phthalate metabolites and lipid peroxidation with sperm mitochondrial DNA copy number and deletions. Environmental Research, 163. doi:10.1016/j.envres.2018.01.023

Jungwirth, A., Giwercman, A., Tournaye, H., Diemer, T., Kopa, Z., Dohle, G., & Krausz, C. (2012). European Association of Urology Guidelines on Male Infertility: The 2012 Update. European Urology, 62(2), 324-332. doi:10.1016/J.EURURO.2012.04.048

Levine, H., Jorgensen, N., Martino-Andrade, A., Mendiola, J., Weksler-Derri, D., Mindlis, I., Pinotti, R., & Swan, S. (2017). Temporal trends in sperm count: a systematic review and meta-regression analysis. Human Reproduction Update, 23(6), 646-659. doi:10.1093/humupd/dmx022

Phillips, N., Sprouse, M., & Roby, R. (2014). Simultaneous quantification of mitochondrial DNA copy number and deletion ratio: a multiplex real-time PCR assay. Scientific reports, 4, 3887. doi:10.1038/srep03887

Rajender, S., Rahul, P., & Mahdi, A. (2010). Mitochondria, spermatogenesis, and male infertility. Mitochondrion, 10(5), 419-428. doi:10.1016/J.MITO.2010.05.015

Shimada, K., Kato, H., Miyata, H., & Ikawa, M. (2019). Glycerol kinase 2 is essential for proper arrangement of crescent-like mitochondria to form the mitochondrial sheath during mouse spermatogenesis. Journal of Reproduction and Development, 65(2), 155-162. doi: 10.1262/jrd.2018-136

Song, G. & Lewis, V. (2008). Mitochondrial DNA integrity and copy number in sperm from infertile men. Fertility and Sterility, 90(6), 2238-2244. doi:10.1016/J.FERTNSTERT.2007.10.0

St John, J.C., Facucho-Oliveira, J., Jiang, Y., Kelly, R., & Salah, R. (2010). Mitochondrial DNA transmission, replication and inheritance: a journey from the gamete through the embryo and into offspring and embryonic stem cells. Human Reproduction Update, 16(5), 488-509. doi: 10.1093/humupd/dmq002

St John, J.C., Jokhi, R.P., & Barratt, C.L. (2005). The impact of mitochondrial genetics on male infertility. International Journal of Andrology, 28(2), 65-73. doi: 10.1111/j.1365-2605.2005.00515.x

Sutovsky, P., Moreno, R.D., Ramalho-Santos, J., Dominko, T., Simerly, C., & Schatten, G. (1999). Ubiquitin tag for sperm mitochondria. Nature, 402(6760), 371-372. doi: 10.1038/46466

Wu, H., de Gannes, M., Luchetti, G., & Pilsner, J. (2015). Rapid method for the isolation of mammalian sperm DNA. BioTechniques, 58(6). doi:10.2144/000114280

Wu, H., Huffman, A., Whitcomb, B., Josyula, S., Labrie, S., Tougias, E., Rahil, T., Sites, C., & Pilsner, J. (2019). Sperm mitochondrial DNA measures and semen parameters among men undergoing fertility treatment. Reproductive BioMedicine Online. doi:10.1016/J.RBMO.2018.10.004

Wu H., Whitcomb, B.W., Huffman, A., Brandon, N., Labrie, S., Tougias, E., Lynch, K., Rahil, T., Sites, C.K., & Pilsner, J.R. (2019). Associations of sperm mitochondrial DNA copy number and deletion rate with fertilization and embryo development in a clinical setting. Human Reproduction, 34(1), 163-170. doi: 10.1093/humrep/dey330

Yang, H., Li, G., Jin, H., Guo, Y., Sun, Y. (2019). The effect of sperm DNA fragmentation index on assisted reproductive technology outcomes and its relationship with semen parameters and lifestyle. Translational Andrology and Urology, 8(4), 356-365. doi: 10.21037/tau.2019.06.22

Zhang, G., Wang, Z., Ling, X., Zou, P., Yang, H., Chen, Q., Zhou, N., Sun, L., Gao, J., Zhou, Z., Cao, J., & Ao, L. (2016). Mitochondrial Biomarkers Reflect Semen Quality: Results from the MARCHS Study in Chongqing, China. PloS one, 11(12), e0168823. doi:10.1371/journal.pone.0168823